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The transcriptionally active P-TEFb that phosphorylates RNA Polymerase II to stimulate general and HIV-specific transcriptional elongation is presumed to exist in a free cyclin T1/CDK9 heterodimer. About half of cellular cyclin T1/CDK9 are also found in an inactive complex containing the 7SK snRNA and the HEXIM1 protein. In this research, I show that the remaining half are associated with Brd4, a bromodomain protein that binds to acetylated histones. In stress-induced cells, the 7SK/HEXIM1-bound cyclin T1/CDK9 is quantitatively converted into the Brd4-associated form for stress-induced transcription. The association with Brd4 is essential to form the transcriptionally active P-TEFb and recruits P-TEFb to transcription templates in vivo and in vitro. Although generally required for transcription, the P-TEFb-recruitment function of Brd4 can be substituted by that of HIV-1 Tat, which recruits cyclin T1/CDK9 for activated HIV-1 transcription. The regulation of the general transcription factor P-TEFb by Brd4, HEXIM1 and 7SK is implicated in regulating cell growth, which may be related to the onset of cancer.

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Introduction

The transcription of protein-coding genes in eukaryotes is performed by RNA polymerase (Pol) II in a dynamic process that can be divided into several stages. Transcriptional elongation has now been recognized as a highly regulated stage capable of not only generating full-length RNA transcripts but also coordinating transcription with other gene expression events such as mRNA capping, splicing and polyadenylation. Several regulatory factors that specifically target the elongation stage have already been identified (Sims et al., 2004). Among these, P-TEFb, containing a heterodimer of CDK9 and its regulatory cyclin T1 (CycT1) subunit (or the minor forms T2 or K), plays a critical role during the transition from abortive to productive elongation (Jones, 1997; Price, 2000). The transcriptional activity of P-TEFb is dependent on the kinase activity of its CDK9 subunit, which hyperphosphorylates the C-terminal domain (CTD) of the largest subunit of Pol II to stimulate the processivity of elongation. Studies using either RNA interference or the highly specific P-TEFb inhibitor flavopiridol have shown that P-TEFb is a global transcriptional elongation factor important for most Pol II transcription in vivo (Chao and Price, 2001; Shim et al., 2002). In addition, P-TEFb appears to be involved in the differentiation program of several cell types, suggesting it may be related to the genesis of transforming events, that may in turn lead to the onset of cancer.

Not only essential for general transcription, P-TEFb is also exquisitely required for HIV-1 gene expression. Tat, a viral-encoded regulatory protein, recruits host P-TEFb to the vicinity of paused Pol II through interacting with the TAR (transactivation-responsive region) RNA element located at the 5' end of the nascent viral transcript. Upon recruitment, the P-TEFb-associated CDK9 kinase phosphorylates the Pol II CTD, leading to the generation of full-length HIV-1 transcripts that are essential for viral gene expression and replication (Mancebo et al., 1997; Wei et al., 1998; Zhu et al., 1997).

In the nucleus, not every CycT1/CDK9 heterodimer displays the P-TEFb transcriptional activity. In fact, about half of the heterodimers in HeLa cells are sequestered in a complex (termed the 7SK snRNP) that also contains the 7SK snRNA and a nuclear protein called HEXIM1 (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003) . While 7SK serves as a molecular scaffold to mediate the interaction of HEXIM1 with CycT1/CDK9, HEXIM1 inhibits the kinase activity of CDK9 and prevents P-TEFb from binding to transcription templates. The association of 7SK/HEXIM1 with CycT1/CDK9 is a dynamic process and can be disrupted by certain stress-inducing agents, such as the global transcriptional inhibitor actinomycin D, kinase/transcription-inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), and DNA-damaging agent UV irradiation (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). At low dosages, these agents have been shown to stimulate the CTD phosphorylation and HIV-1 transcription, which could be directly attributable to the induced disruption of the 7SK snRNP and activation of P-TEFb. Moreover, in cardiac myocytes, hypertrophic signals also cause the disruption of the 7SK snRNP, leading to a global increase in cellular RNA and protein contents and enlargement of heart cells, which is the cause of cardiac hypertrophy (Sano et al., 2002).

Besides 7SK and HEXIM1, a bromodomain protein, Brd4, has recently been identified as a major factor associated with the CycT1/CDK9 heterodimer (Jang et al., submitted). Like all the components of the 7SK snRNP, Brd4 (also known as MCAP) is

also ubiquitously expressed. It belongs to the conserved BET family of proteins that carry two tandem bromodomains and an ET (extra terminal) domain. The bromodomain has been recognized as a functional module in helping decipher the histone code through interacting with acetylated histone tails. Consistent with this view, Brd4 has been shown to bind to acetylated euchromatin through acetylated histone H4 and H3 (Dey et al., 2003). Another noteworthy feature of Brd4 is its ability to associate with mitotic chromosomes (Dey et al., 2000). This property enables Brd4 to tether the Bovine papillomavirus genome to host mitotic chromosomes to ensure viral genome maintenance through an interaction of Brd4 with the viral E2 protein (You et al., 2004).

The recent identification of Brd4 as a major CycT1/CDK9-associated factor prompted us to investigate a potential role of Brd4 in P-TEFb-dependent transcription. Here I showed that Brd4 and HEXIM1/7SK existed in two mutually exclusive CycT1/CDK9-containing complexes and stress-treatment caused a quantitative conversion of the 7SK snRNP into the complex consisting of Brd4 associated with CycT1/CDK9. Importantly, the association with Brd4 is strictly required for forming the transcriptionally active P-TEFb for stimulation of Pol II elongation. Our data demonstrate that Brd4 contributed to general transcription through its recruitment of P-TEFb to transcription templates in vivo and in vitro. However, this role of Brd4 can be functionally substituted by that of the HIV-1 Tat protein, which recruits CycT1/CDK9 for activated HIV-1 transcription.

Body

Brd4 and HEXIM1/7SK exist in two mutually exclusive CycT1/CDK9-containing complexes

We have previously performed immuno-affinity purification of CDK9-associated factors from both HeLa nuclear extract and whole cell lysate. These experiments, however, did not identify Brd4 as a factor associated with the CycT1/CDK9 heterodimer (will be referred to as core P-TEFb from here on). To investigate whether our purification conditions, which routinely used 0.5M KCl in the washing buffer, may have disrupted the binding of Brd4 to core P-TEFb, I examined the stability of the interactions of CDK9 with several protein factors under increasing KCl concentrations. The result showed that the binding of Brd4 to CDK9-f was stable under 0.15 M KCl, but was significantly weakened by 0.25M KCl and abolished by 0.3M KCl. These data reveal the salt-sensitive nature of the binding of Brd4 to core P-TEFb. We further found that Brd4 and HEXIM1/7SK existed in two mutually exclusive CycT1/CDK9-containing complexes. That is, neither HEXIM1 nor 7SK was detected in the Brd4-containing complex. Likewise, only 7SK but not Brd4 was present in the HEXIM1-containing complex.

Stress-induced quantitative transfer of CycT1/CDK9 from 7SK/HEXIM1 to Brd4

HEXIM1 and 7SK have been shown to dissociate from core P-TEFb in cells treated with certain stress-inducing agents such as the global transcription inhibitor actinomycin D, kinase/transcription inhibitor DRB and the DNA-damaging agent UV irradiation. Interestingly, the same treatments consistently increased the binding of Brd4 to core P-TEFb by approximately two-fold, even though the level of Brd4 in NE remained

unchanged before and after the treatments. Given that only ~50% of the total nuclear core P-TEFb are sequestered in the 7SK snRNP, the stress-induced two-fold increase in the Brd4-core P-TEFb binding was significant and represented a quantitative conversion of the 7SK snRNP into the Brd4-containing complex.

Binding of Brd4 to core P-TEFb is essential for transcription

In search of Cdk9 mutants that cannot bind to Brd4, I found an interesting mutant S175D. The S175D-containing core P-TEFb was able to phosphorylate the CTD but unable to bind to Brd4. This provided us with a unique opportunity to examine the contribution of Brd4 to P-TEFb's transcriptional activity. For this purpose, I performed an in vitro transcription assay containing HeLa NE, in which core P-TEFb was immuno-depleted with anti-CDK9 antibodies under high salt conditions to specifically remove the CycT1/CDK9 heterodimer but leave Brd4 behind. Addition of wild-type core P-TEFb fully restored HIV-1 transcription. Interestingly, introduction of the S175D mutant, which was unable to reconstitute with Brd4 in the reaction, largely failed to rescue transcription, even though it was shown to have a comparable level of CTD-kinase activity as the wild-type core P-TEFb.

To further examine the role of Brd4 in P-TEFb-mediated transcription, I performed transcription reactions containing immunodepleted HeLa NE, in which both Brd4 and core P-TEFb were simultaneously removed under high salt conditions. While the addition of either core P-TEFb or Brd4-f alone into the reaction only slightly increased transcription, the introduction of core P-TEFb and Brd4-f together was able to fully restore transcription to the double-depleted NE. Furthermore, the ability of Brd4 and core P-TEFb to interact with each other was crucial to this restoration. Taken together, these data support the model that the binding of Brd4 to core P-TEFb is required for the full transcriptional activity of P-TEFb.

Brd4 recruits core P-TEFb to transcription template in vivo and in vitro

How did Brd4 contribute to P-TEFb's transcriptional activity? In kinase reactions, we did not detect any significant increase in the CDK9 kinase activity by transcriptionally active Brd4 over a broad range of concentrations. However, in a series of experiments involving ectopic expression of either Brd4 or Brd4-specific siRNA, there is a Brd4-dependent increase in the level of CTD phosphorylation on Ser2 in vivo. Since only the un- or hypo-phosphorylated Pol II is recruited to the pre-initiation complex for transcriptional initiation, whereas the Ser2-phosphorylated Pol II is involved in elongation, the different effects of Brd4 on P-TEFb-mediated CTD phosphorylation in vivo and in vitro could be explained by a Brd4-mediated increase in P-TEFb binding to the transcription template, which in turn caused more CTD phosphorylation during elongation.

To test this hypothesis, I performed chromatin immunoprecipitation (ChIP) assay to investigate whether Brd4 may help recruit CDK9 to the integrated HIV-1 LTR-luciferase reporter gene. The result showed that wild-type CDK9 was clearly able to bind more strongly to the promoter, interior and 3' UTR region of the chromatin template than S175D, which failed to bind to Brd4 and displayed signals only at the background level.

To confirm the P-TEFb-recruitment function of Brd4 in vitro, I also examined the effect of Brd4 on the association of CycT1/CDK9 with an immobilized HIV-1 DNA template derived from pHIV+TAR. A significantly reduced association of CDK9 and CycT1 with the HIV-1 template was detected when Brd4 was specifically removed from NE. Taken together, my data indicate a role for Brd4 in recruiting P-TEFb to transcription template both in vivo and in vitro.

Brd4 is not required for Tat-activated HIV-1 transcription in vitro and in vivo

During HIV-1 transcriptional elongation, Tat is known to recruit the CycT1/CDK9 heterodimer to paused Pol II through interacting with the TAR RNA element located at the 5' end of nascent viral transcripts. So next, I asked whether Brd4 is also required for Tat-activated HIV-1 transcription. First, I compared wild-type and the S175D-containing core P-TEFb for their abilities to mediate Tat-transactivation in CDK9-depleted HeLa NE. While the S175D P-TEFb functioned poorly in mediating basal HIV-1 transcription, it surprisingly restored transcription to approximately the same level as that by the wild-type complex when Tat was present in the same reaction. Thus, although S175D P-TEFb was a poor general transcription factor due to its inability to be recruited by Brd4 to the transcription template, it was fully capable of working with Tat and TAR in activating HIV-1 transcription, suggesting that the interaction between Brd4 and core P-TEFb was not required for Tat-transactivation.

Key Research Accomplishment

1. Brd4 and HEXIM1/7SK exist in two mutually exclusive CycT1/CDK9-containing complexes.
2. Stress-induced quantitative transfer of CycT1/CDK9 from 7SK/HEXIM1 to Brd4.
3. Binding of Brd4 to core P-TEFb is essential for transcription.
4. Brd4 recruits core P-TEFb to transcription template in vivo and in vitro.
5. Brd4 is not required for Tat-activated HIV-1 transcription in vitro and in vivo.

Reportable Outcomes

All the key research accomplishments above are reportable.

Conclusion

I show that half of P-TEFb are associated with Brd4, a bromodomain protein that binds to acetylated histones. In stress-induced cells, the 7SK/HEXIM1-bound cyclin T1/CDK9 is quantitatively converted into the Brd4-associated form for stress-induced transcription. The association with Brd4 is essential to form the transcriptionally active P-TEFb and recruits P-TEFb to transcription templates in vivo and in vitro. Although generally required for transcription, the P-TEFb-recruitment function of Brd4 can be substituted by that of HIV-1 Tat, which recruits cyclin T1/CDK9 for activated HIV-1 transcription. The regulation of the general transcription factor P-TEFb by Brd4, HEXIM1 and 7SK is implicated in regulating cell growth, which may be related to the onset of cancer.

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